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Transformation-Defective Rous Sarcoma Virus Mutants with Altered p19 of the *gag* Gene and Their Inhibitory Effect on Host Cell Growth

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Mutants (PH2010, PH2011, PH2012) of Rous sarcoma virus which have a growth-inhibitory effect on chicken embryo fibroblasts were isolated from a temperature-sensitive mutant of the Schmidt-Ruppin strain of Rous sarcoma virus (*ts*NY68). The growth rate of fibroblasts infected with these viruses was about 50 to 60% of that of uninfected fibroblasts. A morphological difference between mutant-infected and uninfected fibroblasts was observed at logarithmic phase but not at stationary phase. Neither the protein $p60^{src}$ nor its associated protein kinase activity was significantly detected by an immunoprecipitation assay in the cells infected with these mutants. Analysis of the unintegrated DNA of the mutant PH2010 showed that a sequence of about 1.4 kilobase pairs at the *src* gene region is deleted. Further examination of the viral structural proteins in infected cells as well as in virions by immunoprecipitation and peptide mapping revealed that the molecular size of the Pr76 *gag* protein of the mutant RSV is smaller than that of the mutant *ts*NY68 because of partial deletion at the p19 *gag* gene. The peptide maps suggest that the deleted region of the altered p19 of the mutant is near the carboxy terminal of p19. The amount of $Prgp92^{env}$ synthesized in the mutant-infected cells was about fivefold more than that in *ts*NY68-infected cells.

It has been well established that the transforming gene products of avian tumor viruses have profound influences on the behavior, morphology, and physiology of the infected host cells (1, 10, 32, 41). On the other hand, it is generally assumed that other viral proteins, such as the *gag* and *pol* gene products, have very little, if any, influence on the cellular function of the infected cells. An exception to this general rule is the apparent cytotoxic effect of subgroup B and D Rous sarcoma virus (RSV) infection (9, 17, 46), which is probably due to the envelopes of these viruses. To date, no effect of the *gag* gene products of RSV on cellular functions has been reported.

The precursor protein to the *gag* products is Pr76, which has a molecular weight of approximately 76,000 (76K). This protein is processed into virion proteins p19, p27, p12, and p15 (1, 7). The function of each *gag* protein is not clear. *gag* protein p15 is believed to be a proteolytic enzyme which processes Pr76 into mature proteins. Protein p27 is thought to form the capsid shell structure, and p12 is found as a nucleoprotein in the virion (7). Most of the p19 in virions exists as a matrix protein near the lipid bilayer of the virions and may interact with the lipids (29, 31) as well as with the glycoprotein of the *env* gene products (34). A minor portion of p19 may be bound to the double-stranded sites of RSV RNA (23, 35), suggesting that p19 may play a role in splicing RSV RNA (24, 45) as well as in the translation of mRNA (43). In this communication, we report transformation-defective mutants whose p19 is significantly smaller than that of their parental strain, RSV *ts*NY68. These mutants (designated as RSV PH2010, PH2011, and PH2012) inhibit the growth of host cells.

MATERIALS AND METHODS

Cells and viruses. Chicken embryonic fibroblasts were prepared and cultured as described previously (19). The following RSV strains were used: a T (transformation)-class temperature-sensitive mutant, Schmidt-Ruppin *ts*NY68 (subgroup A) (16), which was used as a parental virus in this report; a Schmidt-Ruppin wild-type strain of subgroup D (14); the transformation-defective (*td*) mutant Schmidt-Ruppin *td*NY108 (subgroup A) (15); and the *td* mutant Prague *td*PRC (subgroup C) (42). Prague *td*PRC was a generous gift from W. S. Mason (Institute for Cancer Research); the other viruses were kindly supplied by H. Hanafusa (Rockefeller University). Usually, secondary fibroblast cultures were infected at a multiplicity of infection of 1.0. Equivalent amounts of *td* mutants were used after the titer of *td* mutants was measured by a reverse transcriptase assay (13). Culture fluid of cells infected with strain PH2010 had one-

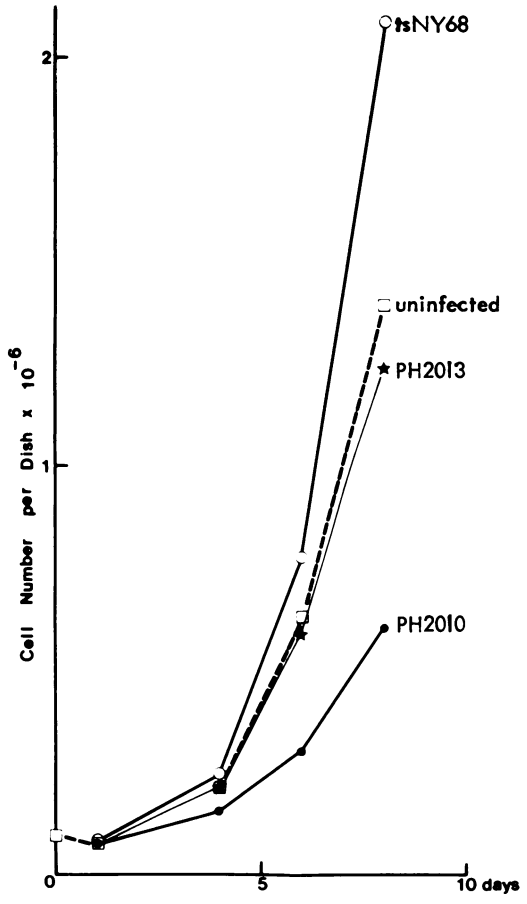

FIG. 1. Inhibitory effect of strain PH2010 on growth rate of fibroblasts. Chick embryonic fibroblasts (20×10^4 cells per 33-mm-diameter dish) were plated, cultured at 36°C for 1 day, and infected with media obtained from uninfected fibroblast cultures (□), *ts*NY68 (○), PH2010 (●), and *td* mutant PH2013 (★), obtained as described in the text. Cells were cultured at 36°C, and media were changed every day. Cell number per dish was determined on days 1, 4, 6, and 8 after the plating. Each plot shows an average of duplicate values. Deviations were within 5% of the average.

third the reverse transcriptase activity of cells infected with *ts*NY68. Focus assays were performed as previously described (30).

Cell growth measurement. Chicken embryonic fibroblasts (20×10^4 cells per 35-mm-diameter dish) were plated, incubated for 1 day, and infected with viruses. The number of cells per dish was measured as described previously (41) with a hemocytometer after the cells were treated with 0.25% (wt/vol) trypsin.

Isolation of mutants. During studies on RSV Schmidt-Ruppin *ts*NY68 (subgroup A) (16), we observed a mutant virus giving significantly small foci. These small foci were often accompanied by surrounding cells with morphologies slightly different from those of the uninfected cells. These surrounding cells were cocultured with normal chicken fibroblasts, and it was found that the culture fluids contained transformation-defective viruses which have an inhibitory effect on the growth of the fibroblasts. These culture fluids were serially diluted up to 10^9-fold. Normal chicken embryo fibroblasts were infected with 1 ml of each of these diluted virus solutions and were cultured at 36°C for 1 month. The reverse transcriptase assay of these infected culture media showed that the endpoint dilution was 10^6-fold. Three independent mutants were separately isolated in this fashion and were called RSV PH2010, PH2011, and PH2012. We consider these viruses to be different because they are independent isolates. The possibility that they originated from one mutant, however, still exists. This question will be answered when the RNA sequences of these mutants are determined. From the same virus stock of strain *ts*NY68, a transformation-defective mutant (PH2013) was further isolated by a method similar to that described above and was used as a control RSV which does not influence cell growth.

Labeling cells and immunoprecipitation of viral proteins. The secondary fibroblasts were plated (3×10^6 cells per 100-mm-diameter dish), infected with virus after the cells attached to the dishes, and cultured at 36°C for 4 days. The virus-infected cultures were treated with 0.25% (wt/vol) trypsin, and the cells were replated (3×10^6 per 100-mm-diameter dish) and cultured at 36°C for 3 days. The cells were then incubated for 2 h at 36°C in 1 ml of medium containing either 100 μCi of [^{35}S]methionine or 500 μCi of $^{32}P_i$. In some cases, the cells were incubated for 1 day in 6 ml of medium containing either 40 μCi of [^{35}S]methionine or 100 μCi of $^{32}P_i$ per ml. After a 2-h labeling period, the cultures were washed twice with STE buffer (0.15 M NaCl, 10 mM Tris-hydrochloride [pH 7.2], 1 mM EDTA) and stored at −70°C. After the 1-day labeling period, the viruses were pelleted from the medium at 25,000 rpm at 0°C for 1 h in a Beckman SW27.1 rotor.

To detect viral proteins by an immunoprecipitation assay, anti-avian myeloblastosis virus (AMV) goat serum (supplied from the National Institutes of Health), tumor-bearing rabbit (TBR) serum (2, 14) prepared in our laboratory (19), and anti-AMV gp85 rabbit serum (no. 19, 09-23-75) (5) (kindly supplied by D. P. Bolognesi of Duke University) were used. Each

TABLE 1. Inhibitory effect of viral strains on growth of fibroblasts[a]

Fibroblasts infected with:	Cells per dish ($\times 10^4$)	Infected cells: uninfected cells
Nothing	187 ± 7	
PH2010	125 ± 7	0.67
PH2011	121 ± 6	0.65
PH2012	132 ± 6	0.71

[a] Chicken embryo fibroblasts (20×10^4 cells per 35-mm-diameter dish) were plated, cultured at 36°C for 1 day, treated with control medium (obtained from uninfected fibroblast cultures) or infected with mutants (PH2010, PH2011, PH2012), and cultured at 36°C. On day 4 after cell plating, the number of cells per dish was determined. Each cell number is the average of duplicate samples. Error range indicates deviations from averages.

pellet of virions and each monolayer of fibroblasts was solubilized on ice for 30 min with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in STE buffer containing 1% Trasylol) or with 1 ml of 1% Nonidet P-40 in STE buffer containing 1% Trasylol (19). The lysates were then incubated with normal serum on ice for 30 min followed by further incubation with formaldehyde-treated *Staphylococcus aureus* Cowan 1 (18). Bacteria and cell debris were removed by centrifugation, and the supernatant fluid was incubated with antisera at 0°C for 1 h followed by incubation with *S. aureus* Cowan 1 again as above. The immunocomplexes were pelleted, washed four times with either RIPA buffer or 1% Nonidet P-40 in STE, and were suspended in 50 μl of sample buffer (3% SDS, 11% glycerol, 5% 2-mercaptoethanol, 70 mM Tris-hydrochloride [pH 6.8]). The samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (20) and subjected to either fluorography (22) or autoradiography with Kodak X-Omat XR-5 films. To estimate the molecular weights of the proteins, the following marker proteins were used (44): myosin (220K), β-galactosidase (130K), phosphorylase *a* (94K), bovine serum albumin (68K), glutamate dehydrogenase (53K), carboxypeptidase (34.6K), and lysozyme (14.3K).

Peptide mapping. After the samples were separated by SDS-PAGE, the protein bands corresponding to either Pr76 in 8% gels or p19 in 12.5% gels were detected by autoradiography and were cut out. The gel slices were homogenized in a buffer (1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% SDS, 0.125 M Tris-hydrochloride [pH 6.8]) and subjected to SDS-PAGE again. The proteins were partially digested with *S. aureus* V-8 protease (2) in the gel (3). After the electrophoresis, the gels were stained and subjected to fluorography (22).

Chemicals. F10 medium, fetal bovine serum, antipleuropneumonia-like organisms, and trypsin (Difco Laboratories 1:250) were purchased from GIBCO Laboratories. SDS (BDH), *S. aureus* V-8 protease (Miles Laboratories), Nonidet P-40 (BDH), [^{35}S]methionine (1,370 Ci/mmol; (Amersham Corp.), $^{32}P_i$ (carrier free; New England Nuclear Corp.), Trasylol (Sigma Chemical Co.), cellulose filter (BA83; Schleicher & Schuell Co.), [α-^{32}P]deoxycytidine triphosphate (2,000 Ci/mmol; Amersham Corp.), and a nicktranslation kit (Bethesda Research Laboratories) were purchased.

TABLE 2. Interference of focus formation of subgroup A virus by mutant viral strains[a]

Fibroblasts infected with:	Foci per dish		
	*ts*NY68		Schmidt-Ruppin wild-type, subgroup D (100 μl)
	0.1 μl	10 μl	
Nothing	114 ± 9	TMTC[b]	242 ± 8
PH2010	0	0	195 ± 27
PH2011	0	0.5 ± 0.5	132 ± 3
PH2012	0	0	157 ± 3

[a] Fibroblasts were infected with PH2010, PH2011, or PH2012, cultured at 36°C for 4 days, replated, and cultured for 4 days more at 36°C. Uninfected or infected cells (40×10^4 cells) were plated on a 35-mm-diameter dish. After 5 h of incubation at 36°C, cells were infected with *ts*NY68 (subgroup A) or Schmidt-Ruppin wild-type, subgroup D, at 36°C for 1 h, overlaid with 2 ml of agar medium per dish, and cultured at 36°C. On day 7, the number of foci per dish was counted. Each value is the average of duplicate samples and the deviation from the average.

[b] TMTC, Too many to count.

TABLE 3. Evidence that growth inhibiton by strain PH2010 was not due to mycoplasma contaminant in the virus preparation[a]

Virus used	Cells per dish ($\times 10^4$)	Infected cells: uninfected cells
None	311 ± 31	
PH2010		
Cultivated without cells	274 ± 26	0.9
Cultivated with cells	151 ± 37	0.5

[a] Medium containing PH2010 virus (5 ml) and fresh F10 medium (5 ml) were incubated at 36°C in a 100-mm-diameter dish with or without 3×10^6 cells per dish. Half of the media was changed every day. Medium was harvested from each culture after 4 days of incubation. The effect on host cell growth was examined as described in the legend to Fig. 1 and was compared with uninfected cell growth. The error range indicates deviation from average.

RESULTS

Inhibitory effect of the mutant PH2010 on growth of chicken embryo fibroblasts. The growth of fibroblasts infected with mutant PH2010 was inhibited (approximately 50%), whereas that of fibroblasts infected with either *ts*NY68 or its *td* mutant PH2013 was not (Fig. 1). In addition, *td*NY108 (another *td* mutant of Schmidt-Ruppin RSV), did not cause any growth inhibition (data not shown). Similar mutants (PH2011, PH2012) were equally effective (Table 1). Although the data presented in Table 1 and Fig. 1 represent durations of 4 to 8 days, separate experiments with much longer periods showed similar results. It should be mentioned that the growth inhibition was not limited to fibroblasts but was observed with other cell types, such as myogenic cells (data not shown). This inhibitory effect on cell growth was not dependent on temperature because the mutant PH2010 inhibited the growth of fibroblasts by about 60% after 5 days of culture at 41.5°C (data not shown). Since subgroups B and D have cytotoxic effects on cells upon virus infection (9, 17, 46), the subgroups of these mutants were examined.

Almost no foci were formed by strain *ts*NY68 (subgroup A) if the cells were first infected with

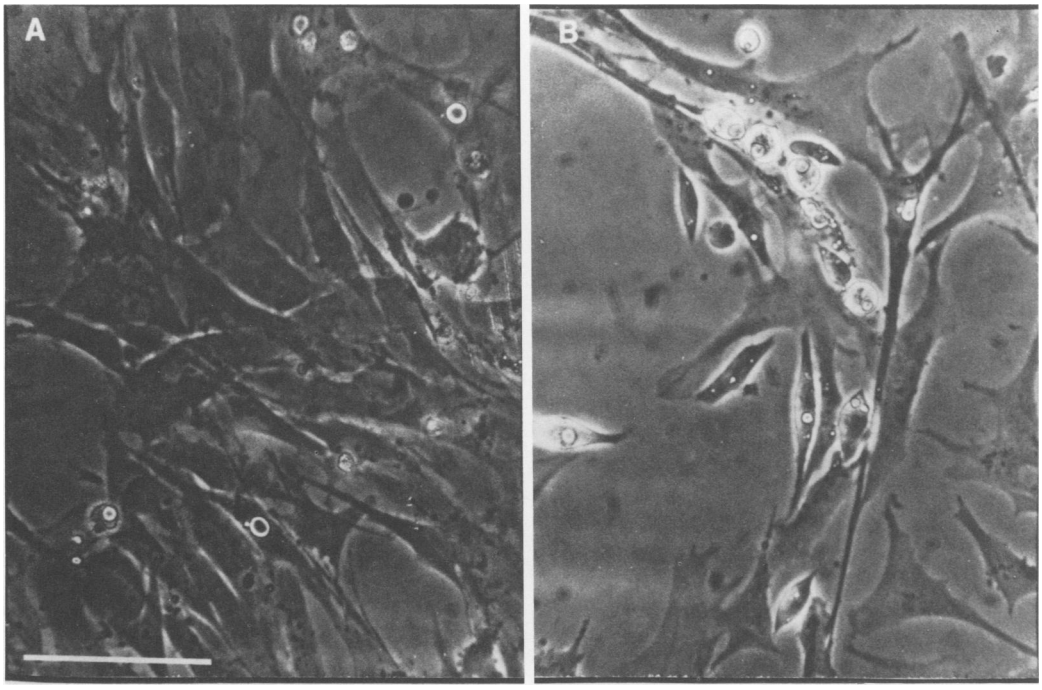

FIG. 2. Morphology of PH2010-infected fibroblasts. Chicken fibroblasts (20×10^4 per 35-mm-diameter dish) were plated, cultured at 36°C, and infected with strain PH2010. Photos were taken on day 6 after plating. (A) Normal uninfected fibroblasts; (B) PH2010-infected fibroblasts. Bar, 100 μm.

E. strain PH2010, Ph2011 or PH2012 (Table 2). On the other hand, these mutant viruses did not interfere with focus formation by the subgroup D Schmidt-Ruppin RSV. We conclude, therefore, that the inhibitory effect on growth observed with PH2010, PH2011, and PH2012 is not due to contamination by subgroup B or D viruses.

That inhibition of growth is not due to contamination with mycoplasma-like organisms is shown in Table 3. In this experiment, the virus solution alone was incubated in a culture medium to allow presumed mycoplasma-like organisms to grow without fibroblasts. The solution was then applied to chicken embryo fibroblasts. It is clear from this table that no appreciable inhibitory effect was observed when the virus solution alone was incubated to allow the mycoplasma, if it existed, to multiply. In a separate test for pleuropneumonia-like organisms (conducted by the Institute for Medical Research), the virus solution did not have any microbiological contaminants (data not shown).

TABLE 4. [2-^{3}H]deoxyglucose uptake of RSV mutant-infected fibroblasts[a]

Fibroblasts infected with:	[2-^{3}H]deoxyglucose uptake (cpm/100 μg of protein)	Infected cells: uninfected cells
Nothing	5,300 ± 100	
PH2010	8,200 ± 600	1.5
PH2012	7,200 ± 300	1.4
*ts*NY68	22,900 ± 700	4.3

[a] Fibroblasts were infected with strain PH2010, PH2012, or *ts*NY68, cultured at 36°C for 4 days, replated, and cultured at 36°C for an additional 4 days. After trypsinization, 40×10^4 cells per 35-mm-diameter dish were plated and cultured at 36°C for 3 days, and the level of [2-^{3}H]deoxyglucose uptake was examined (28). Each value shows an average of duplicate samples with error ranges (deviation from average).

Lack of *src* gene expression in chick embryo fibroblasts infected with the mutants. In the experiment shown in Fig. 2, the morphology of chick embryo fibroblasts infected with the mutant PH2010 was compared with that of uninfected fibroblasts. It appeared that more spindle-shaped and rounded fibroblasts were frequently observed among the cultures infected with PH2010. These were only subtle differences. This subtle difference could only be observed when the cells were not confluent. It was also noted that these mutants did not form foci under the usual focus assay conditions (data not shown). There was not discernible morphological change of cells infected with the control *td* strain PH2013.

The lack of expression of *src* gene activity is not limited to a lack of dramatic change in morphology. The glucose uptake of chicken embryo fibroblasts infected with the mutants was only slightly elevated whereas under similar conditions, the glucose uptake of *ts*NY68-transformed cells at 36°C was elevated at least fourfold (28) (Table 4).

In confirmation of the conclusion from cell biological studies, the experiment shown in Fig. 3 indicates that no appreciable *src* gene product ($pp60^{src}$) (2) was detected in the extract of chicken embryo fibroblasts infected with the mutants PH2010, PH2011, or PH2012. In this experiment, the infected cells were labeled with [^{35}S]methionine or $^{32}P_i$, and the labeled proteins were precipitated with TBR serum, which contains an antibody against the *src* gene product as well as viral proteins. It is noted in this figure that a significant amount of the *src* gene product, $pp60^{src}$, labeled with [^{35}S]methionine or $^{32}P_i$ was detected in the control cells transformed by strain *ts*NY68. In contrast, parallel cultures infected with the mutants had neither appreciable radioactive bands corresponding to the position of $pp60^{src}$ nor any smaller protein which might be a part of $pp60^{src}$. Furthermore, the protein kinase activity was examined in PH2010-infected cells since this is the most sensitive method for detecting the *src* protein (4, 26). It is clear that there was no significant protein kinase activity in PH2010-infected fibroblasts (Table 5).

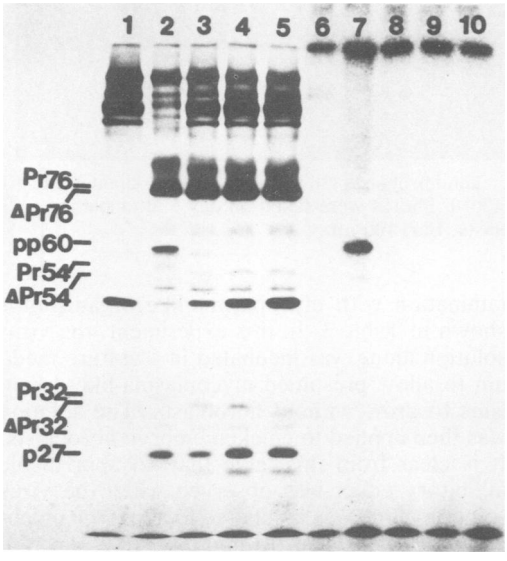

FIG. 3. Viral proteins in mutant-infected fibroblasts. Fibroblasts infected with *ts*NY68 (parental virus) or mutants (PH2010, PH2011, or PH2012) were labeled at 36°C for 2 h with [^{35}S]methionine (lanes 1 through 5) or $^{32}P_i$ (lanes 6 through 10) as described in the text. The cells were solubilized with RIPA buffer and subjected to immunoprecipitation with TBR serum. The immunoprecipitates were analyzed by SDS-PAGE (10%) and autoradiography. Uninfected fibroblasts, lanes 1 and 6; fibroblasts infected with *ts*NY68, lanes 2 and 7; with PH2010, lanes 3 and 8; with PH2011, lanes 4 and 9; with PH2012, lanes 5 and 10. The protein moving slightly slower than Pr76 is not the *env* precursor and is probably not related to RSV proteins (lanes 2 through 5) because precipitation by anti-*env* antibody did not result in similar patterns (see Fig. 5). In addition, with the SRA2 strain of RSV, which has a smaller *env* protein than pH2010, a band identical to that shown in this figure was observed. If this band was the *env* precursor, one would expect it to be shorter. We do not now know, however, the identity of this band.

TABLE 5. Lack of protein kinase activity associated with $p60^{src}$ in PH2010-infected fibroblasts[a]

Fibroblasts	Protein kinase activity to immunoglobulin G
No cells	0
Uninfected	0
Infected with:	
*ts*NY68	2,130
*td*NY108	30
PH2010	40

[a] Fibroblasts infected with strain *ts*NY68, *td*NY108, or PH2010 were prepared as described in the text. Cells were solubilized with 1% Nonidet P-40. After centrifugation, each cell lysate (200 μg protein) was subjected to immunoprecipitation with TBR serum. Immunoprecipitates were incubated in the protein kinase assay mixture containing [γ-^{32}P]ATP as described previously (4) and subjected to SDS-PAGE. The immunoglobulin G heavy-chain portion of the gel detected by autoradiography was cut out, and its radioactivity was measured with a liquid scintillation counter. As negative controls, a solution without cell extract and another solution with uninfected cell extract were treated identically. The values of these controls are 320 and 270 cpm, respectively. The value 220 cpm was used as a background count. Each value shown in the table has been adjusted for this background value and is shown as counts per minute 200 μg of protein (27) of cleared cell lysate.

To ascertain whether these mutants are *td* mutants, unintegrated RSV DNAs were recovered from infected cells by the fractionation method of Hirt (11) and digested with *Eco*RI. These samples were analyzed by the Southern blotting method (39) followed by hybridization with [^{35}P]DNA of RSV representing total genome (6), as described previously (33). The profile of restriction enzyme digests of strain PH2010 was quite different from that of the parental strain, *ts*NY68 (Fig. 4). The 3.0-kilobase band was clearly missing, but a new band corresponding to 1.6 kilobases appeared. These

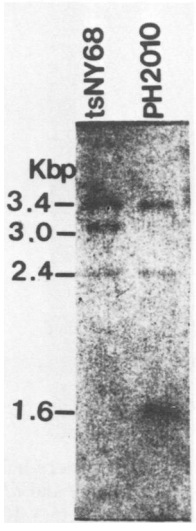

FIG. 4. Analysis of unintegrated DNA in PH2010-infected fibroblasts. Secondary chicken fibroblasts were plated (3×10^6 cells per 100-mm-diameter dish) and infected with strain *ts*NY68 or the PH2010 mutant as described in the text. Cells cultured at 36°C for 1 day after the infection were used in this experiment. Unintegrated RSV DNA was obtained by the method of Hirt (11), followed by pronase digestion, phenol-chloroform extraction, ribonuclease digestion, and ethanol precipitation as described previously (33). The DNA was digested with restriction enzyme *Eco*RI, followed by electrophoresis on a slab gel of 1% (wt/vol) agarose. The DNA was transferred to a nitrate cellulose filter by the method of Southern (39) and subjected to hybridization with ^{32}P-labeled RSV DNA prepared by nick translation of cloned SRA-2 DNA (6) (a generous gift from J. M. Bishop). Viral DNA was detected by autoradiography. Molecular size of DNA was determined by DNA markers digested with *Hin*dIII.

results show that a portion of the *src* gene region is missing from PH2010.

Overproduction of *env* protein in cells infected with strain PH2010. Chicken fibroblasts infected with RSV strain PH2010 produced a larger amount of *env* protein than those infected with RSV strain *ts*NY68 (Fig. 5). In this experiment, cells were infected with *ts*NY68, PH2010, or *td*NY108. RSV *td*NY108 is a *td* RSV with a deleted *src* gene (15). Proteins labeled with [^{35}S]methionine were precipitated from the cell lysates and analyzed by SDS-PAGE. It is clear from Fig. 5 that the production of *env* protein by PH2010-infected cells was two- to threefold higher than that of those infected with either RSV *td*NY108 or *ts*NY68. No such overproduction of viral *gag* protein was observed (Fig. 3).

***gag* proteins of strain PH2010 are different from those of parental and other *td* strains.** In the experiment shown in Fig. 6, viral structural proteins were labeled with [^{35}S]methionine and analyzed by gel electrophoresis. It is clear from this figure that the structural protein p19 of strain PH2010 was not identical to that of strain *ts*NY68 (Fig. 6A). The parental RSV strain had the p19 consisting of two components, in confirmation of previous reports (8). On the other hand, PH2010 had no bands corresponding to those two bands of p19, and a new band appeared migrating significantly faster than the control p19 proteins. The fact that this protein from PH2010 migrated faster than either the two

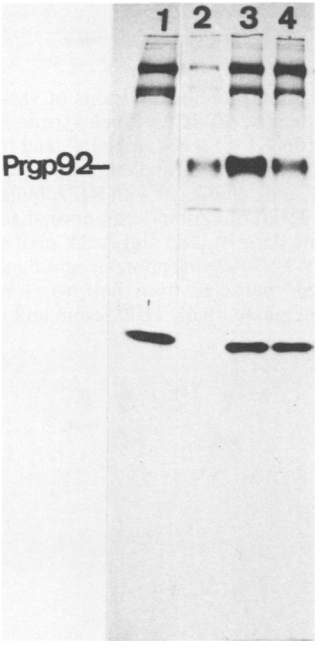

FIG. 5. PH2010-infected fibroblasts producing larger amounts of the *env* precursor. Fibroblasts infected with strain *ts*NY68, PH2010, or *td*NY108 were labeled with 4 ml of minimal essential medium containing 5% (vol/vol) fetal calf serum and 100 μCi of [^{35}S]methionine in a 100-mm-diameter dish at 36°C for 3 h. Cells were solubilized with RIPA buffer, and the cell lysates (4×10^6 cpm) were subjected to immunoprecipitation with anti-gp85 followed by SDS-PAGE and fluorography. Gels show proteins from uninfected fibroblasts (lane 1) and fibroblasts infected with *ts*NY68 (lane 2), PH2010 (lane 3), and *td*NY108 (lane 4). Gels corresponding to Prg92^{env} were cut out, solubilized in H_2O_2 at 60°C, and counted. Samples of cells infected with *ts*NY68, PH2010, and *td*NY108 had 1,400, 7,200 and 2,700 cpm of radioactivity, repectively. Note that a constant amount of radioactivity of each sample was applied. The specific radioactivities of protein per unit of DNA weight were approximately similar for *td*NY108 and pH2010 but approximately 20% more for *ts*NY68. Therefore, even on the basis of per unit of DNA weight, cells infected with pH2010 produced more *env* protein than cells infected with *ts*NY68 did.

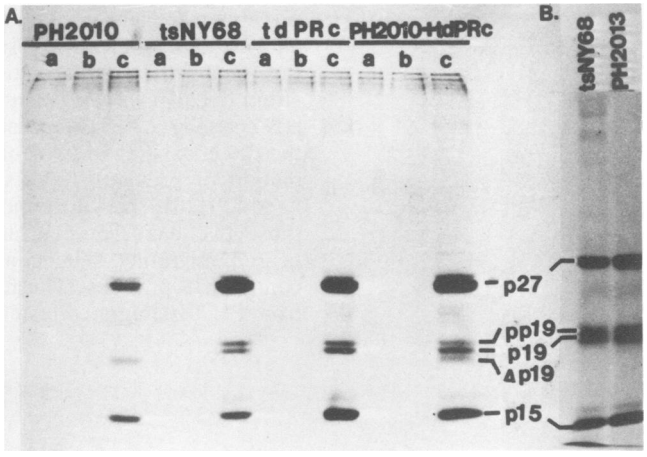

FIG. 6. Viral proteins in virions of various RSV mutants. (A) Chicken fibroblasts were infected with strain PH2010, *ts*NY68, *td*PRC (a Prague-strain RSV of subgroup C), or a mixture of PH2010 and *td*PRC. They were cultured at 36°C for 1 week, replated, and further cultured at 36°C for 1 week. After cells (5×10^6 per 100-mm-diameter dish) were plated and cultured at 36°C for 1 day, viruses were labeled at 36°C with [^{35}S]methionine for 24 h, pelleted, and solubilized with RIPA buffer. Acid-insoluble cpm of each sample (2×10^5) were immunoprecipitated with 2.5 μl of various sera, normal goat serum (lane a), anti-AMV goat serum preabsorbed with Prague A RSV virions (lane b), and anti-AMV goat serum (lane c). Each immunoprecipitate was subjected to polyacrylamide gel (12.5%) electrophoresis and fluorography. pp19 designates phosphorylated p19. (B) [^{35}S]methionine-labeled RSV particles from fibroblasts infected with *ts*NY68 or with a control *td* mutant PH2013 were immunoprecipitated with TBR serum and analyzed by SDS-PAGE as described above.

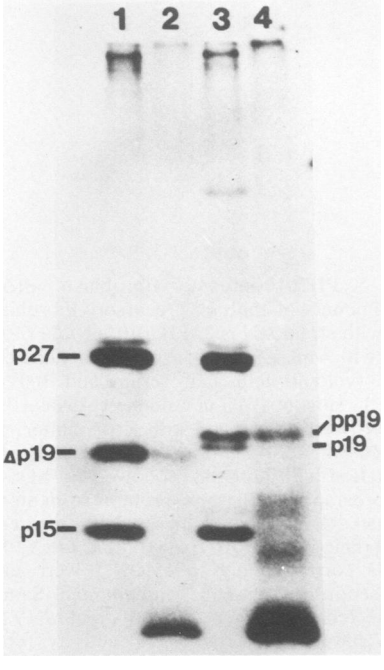

FIG. 7. Phosphorylated p19 of strain PH2010. Fibroblasts infected with PH2010 *ts*NY68 were labeled with [^{35}S]methionine or $^{32}P_i$ at 36°C for 1 day, and the labeled viruses were subjected to immunoprecipitation with anti-AMV goat serum followed by SDS-PAGE and fluorography. Lane 1, PH2010 virions labeled with [^{35}S]methionine; lane 2, PH2010 virions labeled with $^{32}P_i$; lane 3, *ts*NY68 virions labeled with [^{35}S]methionine; lane 4, *ts*NY68 virions labeled with $^{32}P_i$.

bands of p19 of either *ts*NY68 or *td*PRC can be demonstrated by analysis of mixed viruses of PH2010 and *td*PRC. In the mixed sample, three bands were observed, one corresponding to the p19 of PH2010 (Δp19) which moved fastest and two others corresponding to the p19 of *td*PRC. The Δp19 of PH2010 could not be precipitated with either normal serum or anti-AMV goat serum previously absorbed with disrupted virions (Fig. 6A), suggesting that the Δp19 of PH2010 is an altered virion protein. The other two mutants (PH2011 and PH2012) had a similarly altered p19 (data not shown). Figure 6B shows that a control *td* mutant PH2013 isolated from the virus stock of *ts*NY68 had a normal p19. It should be noted that PH2013 had no effect on cell growth (Fig. 1A), yet all three independently isolated mutants had inhibitory effects on host cell growth (Table 1).

It has been shown that protein p19 exists in both a phosphorylated form and a nonphosphorylated form in virions (8, 21, 37). In the experiment shown in Fig. 7, PH2010, *ts*NY68, and virions were labeled with $^{32}P_i$ or [^{35}S]methionine, and the labeled viral proteins were examined. It is clear from this figure that the p19 of *ts*NY68 has a phosphorylated form as well as a non-phosphorylated form, but only a phosphor-

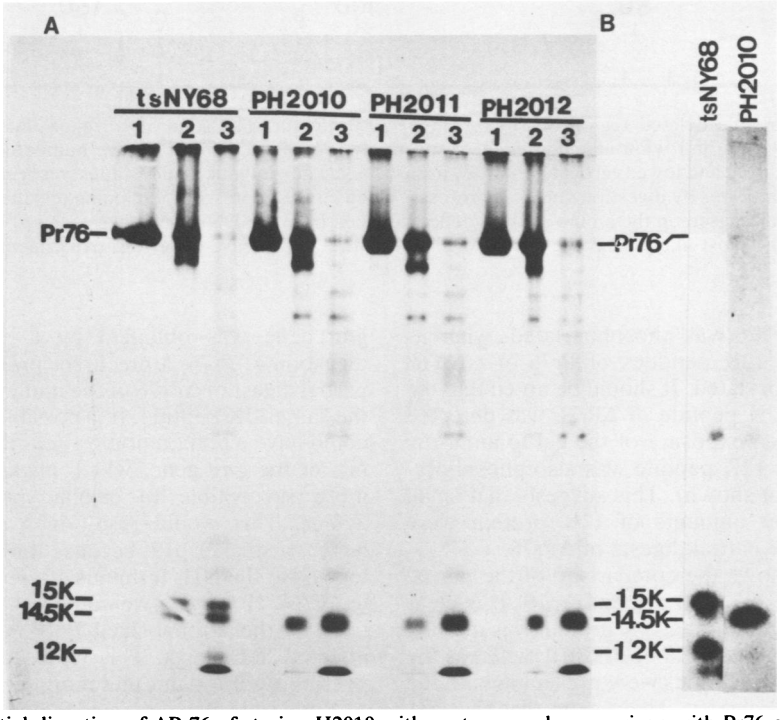

FIG. 8. Partial digestion of ΔPr76 of strain pH2010 with protease and comparison with Pr76 of tsNY68. (A) From RSV-infected fibroblasts labeled with [^{35}S]methionine at 36°C for 2 h, ΔPr76 and Pr76 were isolated by immunoprecipitation with TBR serum and SDS-PAGE (8% gel). Each sample was digested with 0 (lane 1), 0.005 (lane 2), or 0.05 (lane 3) μg of V-8 protease (12) and was subjected to SDS-PAGE (12.5%) and autoradiography. (B) From infected fibroblasts labeled with $^{32}P_i$ at 36°C for 2 h, ΔPr76 and Pr76 were isolated, partially digested with V-8 protease (0.05 μg), and analyzed as in (A).

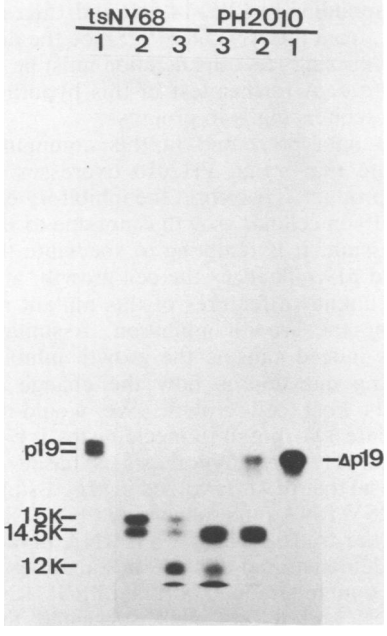

FIG. 9. Partial digests of p19 of strain PH2010. From *ts*NY68 and PH2010 virions labeled with [^{35}S]methionine, p19 and Δp19 were isolated and partially digested with 0 (lane 1), 0.005 (lane 2), or 0.05 (lane 3) μg of V-8 protease followed by SDS-PAGE (12.5%).

ylated form of the p19 (Δp19) of PH2010 was observed.

Partial proteolytic digestion of ΔPr76 and Δp19. It was noted in Fig. 3 that the *gag* precursor protein of strains PH2010 and PH2012, Pr76, is slightly smaller than the Pr76 of strain *ts*NY68. We designate this slightly smaller precursor ΔPr76. In further confirmation of the notion that PH2010 and the two other mutants, PH2011 and PH2012, have altered *gag* proteins, we have isolated ΔPr76 labeled with [^{35}S]methionine or $^{32}P_i$ from the cells infected with the mutants and performed fingerprinting of the partial proteolytic digests of these proteins. It is clear (Fig. 8A) that most of the partially digested proteins derived from Pr76 of *ts*NY68 correspond to those of ΔPr76 derived from the mutants. However, it is noted that *ts*NY68 showed bands corresponding to 12K and 15K proteins, whereas these bands were missing in the digests of mutants. Figure 8B shows that only the 14.5K

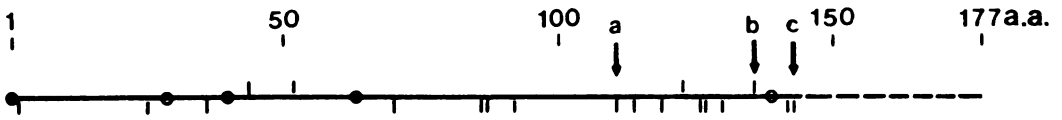

FIG. 10. Possible deleted region of p19 of the PH2010 mutant. The solid and broken lines represent the regions of p19 with and without methionine, respectively. Amino acid residues are numbered from NH_2 to COOH ends. Upper and lower vertical bars show aspartic acid and glutamic acid residues, respectively. They are possible cleavage sites by digestion with V-8 protease. Open circles represent methionine residues. These amino acid positions are based on the amino acid sequence deduced from the DNA sequence of the p19 gene (40). The arrows show the most likely cleavage site by digestion with V-8 protease under our experimental conditions.

protein of ΔPr76 was phosphorylated, whereas the 15K and 12K peptides of Pr76 of *ts*NY68 were phosphorylated. It should be noted that the ^{35}S-labeled 12K peptide of ΔPr76 was detected after prolonged exposure of the gel to an X-ray film, and this 12K peptide was also phosphorylated (data not shown). This suggests that small but significant amounts of 12K protein were present in the partial digests of ΔPr76.

Figure 9 shows the comparison of the partial digests of the parental p19 and Δp19. It is clear from this figure that the 15K protein was missing from the digests of Δp19 of PH2010, whereas the 12K and 14.5K peptides were present in both the p19 and Δp19 digests. The reason that the 12K peptide was present in Δp19, although not apparent in ΔPr76 digests (Fig. 8), is probably the difficulty of digesting ΔPr76 owing to its configuration.

DISCUSSION

It has been reported that some of the recombinant viruses between the subgroup E virus and the exogenous RSV have a smaller (p19β) and a larger (p19α) p19 protein (37, 38). The interaction of p19β with RSV RNA was weaker than with the control p19 (24). These recombinant viruses may be similar to the mutants reported in this communication in that both involve alterations of p19. However, no clear-cut effect of these recombinant viruses on host cell growth has been reported. In fact, a protein similar to p19α was occasionally observed during our analysis. A pulse-chase experiment on this virion protein and a peptide analysis suggest that this is the precursor of p19 (K. Hsia, A. Tanaka, and A. Kaji, unpublished observation). PH2010 and related mutants may, however, be different from these recombinants because our mutants were isolated in a completely different way.

Partial proteolytic digestion of the altered p19 (Δp19) of strain PH2010 strongly suggests that the mutant has a partial deletion of the p19 gene. Thus, two of the digested peptides of Δp19 appear to be identical to those of the p19 of the parental strain, whereas one 15K peptide is clearly missing from Δp19. Supporting evidence for the notion that PH2010 has a deletion in the *gag* gene was obtained by a similar partial digestion of Pr76, a precursor protein to p19. A partial digest of ΔPr76 of the mutant was missing the same 15K peptide. It is possible that PH2010 could have a point mutation near the NH_2 terminus of the *gag* gene, which might make ΔPr76 more susceptible to cellular proteolytic enzymes. This would result in a smaller Pr76, hence, a smaller p19, because the p19 protein is located at the NH_2 terminus of the Pr76 polyprotein (36). However, we could not detect even a trace of the normal-sized Pr76 in the PH2010-infected fibroblasts. This tends to rule out the possibility of a point mutation.

A possible deleted region of Δp19 could be determined from data obtained by partial proteolytic digestion of p19 (Fig. 9) and amino acid sequence data deduced from the DNA sequence of Schmidt-Ruppin RSV (subgroup A) (40). Figure 10 shows the possible cleavage sites in Δp19 for V-8 protease under our experimental conditions. Cleavage at a, b, or c produces peptides corresponding to 12K, 14.5K, and 15K, respectively, from the *ts*NY68 p19. Since the digest of p19 is missing 15K, the deletion must be around the site c. A further test of this hypothesis by DNA sequencing is in progress.

The data presented in this communication indicate that strain PH2010 expresses no *src* gene product. Therefore, the inhibitory effect of PH2010 on cellular growth is not due to *src* gene expression. It is tempting to speculate that the altered p19 influences the cell growth, although other unknown features of this mutant may be causing the growth inhibition. Assuming that p19 is indeed causing the growth inhibition, a puzzling question is how the change in p19 inhibits host cell growth. We would like to postulate two possible mechanisms for further studies. The first hypothesis is based on the proposal that p19 is involved in processing genomic RSV RNA into subgenomic RNAs (23, 24) or in the translation of RSV RNA (43). It has been demonstrated that p19 has a strong affinity to the double-stranded portion of RSV RNA (23, 24, 35), and it has been suggested that the complex of p19 and RNA is resistant to some processing enzyme encoded by host cells. If one

assumes that the altered p19 (Δp19) has an abnormally strong affinity to the double-stranded portions of the host RNA, the altered p19 might interfere with the proper processing of the host RNAs (25) or the efficient translation. The second hypothesis is based on the observation that most of p19 is a matrix protein near the lipid bilayer in the virion (29) and presumably has a strong affinity to lipids (31). Again, if the altered p19 has an unusual affinity to the lipid portion of host cell membranes, it could interfere with host cell growth. In this connection, one must not disregard the fact that the relative amounts of the *env* precursor $Prgp92^{env}$ of PH2010 are higher than those of the parental strain. This may be due to the efficient splicing (24) or the translation of *env* mRNA. Since the *env* protein has an affinity for the cell membrane, overproduction of this protein may cause abnormal membrane behavior. The latter hypothesis is consistent with slight but noticeable changes in cell morphology and slight increases of the 2-deoxyglucose uptake upon infection with strain PH2010.

Although no evidence for it is available, there is a third possibility that should not be dismissed. It is possible that the mutants we isolated have another lesion resulting in the defect in transformation and growth inhibition. This would mean that the *src* gene is deleted in such a way as to cause growth inhibition. Final proof that the alteration in p19 is causing growth inhibition awaits the complete sequencing of these mutants. Work is in progress toward this goal.

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